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Novel Anti-malarial Atovaquone Prodrug: Synthesis, Characterization and *in vitro* Kinetic Study

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Jerusalem–Palestine

1437/2016

الاهداء

اتقدم بأسمى آيات الشكر والامتنان والتقدير والمحبة إلى الذين حملوا أقدس رسالة في الحياة...
و مهدوا لنا طريق العلم والمعرفة...
إلى جميع أساتذتي الأفاضل.
واخص بالشكر والتقدير البروفسور رفيق قرمان, غلى جهوده وعلى ما افاضه علي من معرفة خلال رحلتي البحثيه.

ولا بد لي من وقفة اجلال وحب وتقدير.....

الى من احمل اسمه بكل فخر, ويرتعش قلبي لذكره. الى من جرع الكاس فارغا ليسقينا قطرة حب....

والى من ارضعتني الحب والحنان, رمز النقاء والصفاء وبلسم الشفاء, الى اعظم وانقى انسانه....

يا من حصدا الاشواك عن دروبنا ليمهدا لنا طريق العلم....

ابي العزيز (وضاح), وامي الغاليه (جميله).

الى زوجي الغالي ورفيق دربي سفيان.

الى قرة عيني وفلذة كبدي ابنتي كرمل.

الى القلوب الطاهره الرقيقه ورياحين الحياه...

اخي محمد واخواتي الحان, دانيه, ايمان و هبه.

الى النفوس البريئه و بسمة الحياه وربيعها ابناء شقيقاتي وشقيقي , شكري, سليمان وملك.

اهديكم جميعا هذا البحث, راجية من المولى عز وجل ان يتقبله مني وان يجد القبول والنجاح

Declaration

I certify that the thesis submitted for the degree of master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not be submitted for a higher degree to any other university or institution

Signed: ……..

Bisan Waddah Amin Alfattash.

Date: 5/1/2016

ABSTRACT:

Malaria is a global public health problem, resulting in tow million deaths per year .The majority of death cases are due to the most severe form of malaria caused by *Plasmodium falciparum.* As a result, efforts were directed toward the development of new effective medications intended for the treatment of this endemic disease. Atovaqoune is a new treatment option, showed an improvement in malaria treatment. Although Atovaqoune is an effective medication, it has its own limitations.

Atovaqoune is highly lipophilic compound (water solubility < 0.2 µg/ mL), has low water solubility and low absorption, hence low bioavailability (< 10% in the fasted state). Accordingly, increasing atovaqoune aqueous solubility will improve its pharmacokinetic profile in particular bioavailability, thus improving its effectiveness and ability to administer the drug through different routs of administration.

So that, our goal was to develop atovaqoune prod rug that possesses increased aqueous solubility by linking water soluble moiety to the 3- hydroxyl group, via chemical synthesis. Purification techniques including extraction, re-crystallization and column chromatography were used. Identity confirmation was done using, IR, NMR and LC/MS. Based on purity and identity results; in *vitro* kinetic studies using the HPLC instrument were performed at pH 2.2, 5.5 and 7.4.

ATQ succinate (ProD1) has been successfully synthesized, purified and evaluated. T1/2 of ProD1 at pH 2.2, 5.5 and 7.4 is 28.8 days, 2.2 days, 3.2 days, respectively.

It can be concluded from these data, that ProD1 is converted into ATQ in pH dependent manner, and the hydrolysis of the prodrug follows first order kinetics, as the data plotted gives a straight line, and the K_{obs} is nearly constant. Concisely, modifying atovaqoune structure may result in enhancing its pharmacokinetic profile mainly absorption into body tissues, consequently increasing efficacy and ability to formulate atovaqoune in different dosage forms.

Keywords: Malaria, *drug resistance, atovaquone, bioavailability, prodrugs.*

Contents

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Abbreviations

ATQ: Atovaquone.

DFT: Density functional theory.

FT-IR: Fourier Transform Infra- Red.

HPLC: High Performance Liquid Chromatography.

K_{obs}: The observed hydrolysis rate constant.

LC/MS: Liquid chromatography/ Mass spectroscopy.

MS: Mass Spectroscopy.

M/z: Mass to charge ratio.

NMR: Nuclear Magnetic Resonance.

PK: Pharmacokinetic.

ProD1: ATQ succinate prodrug.

RBF : Round bottom flask.

R_f :Retention Factor.

SMEDDS: Self micro-emulsifying drug delivery systems.

$T_{½}$: Half-life.

TLC: Thin layer chromatography

Introduction

Chapter One

Introduction

1.1 Background

1.1.1 Malaria

Malaria is a global public health problem, affecting 40% of the population (300 million clinical cases annually) and causes~ 2 million deaths per year [1, 2].

Most of disease cases are found in tropical Africa, Latin America, Southern Asia and Oceania [3]. World Health Organization (WHO) assesses that 81% of cases and 91% of deaths are found to be in WHO African regions. Children under 5years old and pregnant women are the most severely affected. This protozoan disease is caused by 5 parasites species of the genus *Plasmodium* that affect humans (*P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*) [4]. Most of death cases are caused by the most severe form *P. falciparum*, which dominate in Africa and to which most drug-resistant cases are attributed. Malaria is transmitted to humans via the bite of infected female mosquito of anopheles species. Malaria can exist, in a mild form that most commonly associated with flu-like symptoms; fever, vomiting, and general malaise. While in the sever form caused by *P. falciparum*, a nervous, respiratory and renal complications frequently coexist due to serious organ failure [5]. Despite of being serious infectious disease, Malaria is a treatable and preventable illness and a number of treatments are already available [4].

1.1.1.1 Malaria treatment medications

A brief description of the major therapeutic groups of malaria including chloroquine, antifolates, artemisinins and atovaquone are provided below.

1.1.1.1.1 Chloroquine

Chloroquine a 4-aminoquinoline, act by accumulating inside the digestive vacuole of the infected red blood cell, where it makes complexes with toxic heme moieties and disturbs the detoxification mechanisms that involve heme sequestration into an inert pigment called hemozoin[6]. It is an inexpensive drug used to prevent and treat malaria for decades. However, the emergence of chloroquine resistance in the vast majority of malaria-endemic countries, and the association of tinnitus and central nervous system toxicity with chloroquine treatment limit its use [7] [8].

1.1.1.1.2 Antifolates

Currently used antifolate combinations of sulfadoxine- pyrimethamine and sulfalene-pyrimethamine have long elimination half-lives, 81 hours for sulfadoxine, 62 hours for sulfalene and 116 hours for pyrimethamine [9], [10]. This has both advantages and disadvantages. On the one hand, it allows single-dose therapy and persistence of the drugs at effective blood levels might protect the patients from reinfections after cure of the initial disease. On the other hand, the latter would be only useful in high transmission areas and the slow elimination favors the selection of resistant parasites[11]. There is also concern with adverse reactions to long-acting sulfonamides [12], especially in subjects concomitantly infected with human immunodeficiency virus (HIV) infections [13].

As indicated from the name ‘antifolates’, they antagonize the action of folic acid by inhibiting dihydrfolate reductase (DHFR) enzyme, hence inhibiting cell division. [14]

1.1.1.1.3 Artemisinin

Replacing unsuccessful medications (chloroquine), with well tolerated artesunate monotherapy and artemisnin combinations resulted in decrease in malaria mortality and morbidity [15] . Artemisinin are commonly used in Southeast Asia [16].

The mechanism of action of these compounds appears to involve the heme-mediated decomposition of the endoperoxide bridge to produce carbon-centered free radicals [17]. In spite of their effectiveness, artemisinin resistance appears in several areas mainly in Pailin and western Cambodia. Moreover, it is associated with reduced cure rates [18].

It is worth noting that antimalarial drug resistance escalates to the major therapeutic groups used in malaria treatment, which constitutes a major threat to the global malaria control [19]. This can be attributed to the fact that malaria control has significantly dependent on a limited number of chemically related drugs, such as the quinolone or the antifolate groups, which are overused in poor countries due to their low price[16].

Practice has shown that resistance ultimately shortens the life span of antimalarial drugs. Accordingly, this emphasizes the urgent need to develop alternative medications with a novel chemical structure and mechanisms of action to treat and prevent malaria in one hand, and on the other hand to develop strategies to avoid resistance when new drugs are introduced [16].

In view of that, efforts were directed toward developing, novel compounds with novel mechanisms of action to maintain an effective malaria control**.**

1.1.1.1.4 Atovaquone

Atovaquone (ATQ) is a hydroxynaphthoquinone figure (1.1). Naphthoquinones are known to have antimalarial, anticoccidial and antitheilerial activity [20]. ATQ is relatively a new treatment option, that has a broad antiprotozoan activity including *Plasmodium spp* [21], it has a novel mechanism of action, acts by inhibition of the electron transport system at the level of cytochrome bc1 complex [22] figure(1.2). In malaria parasites, the mitochondria act as a sink for the electrons generated from dihydrorotate dehydrogenase; an essential enzyme for pyrimidine biosynthesis; Inhibition of electron transport by ATQ leads to dihydrorotate dehydrogenase inhibition resulting in reduced pyrimidine biosynthesis and

concomitantly, parasite replication [23]. This is because parasites depend on de novo production of pyrimidines and have no salvage pathway; in contrast to humans, thus the final outcome is the prevention of parasite replication [24]. It is mentioned in the literature; that protozoan electron transport inhibition was about 1000-fold more sensitive than were mammalian and avian mitochondria [25].

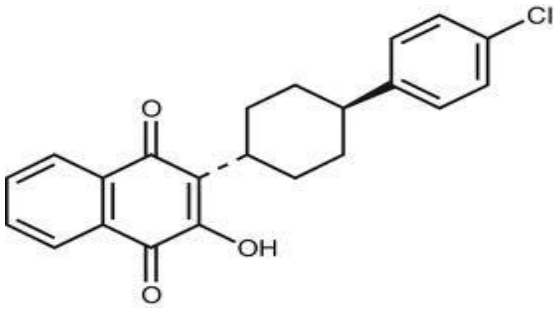


Figure 1.1: Atovaquone chemical structure.

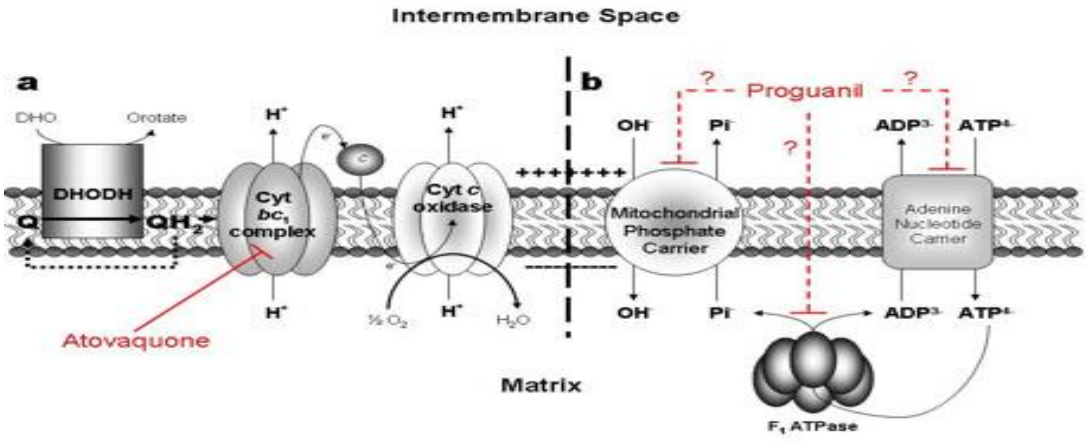


Figure 1.2: ATQ mechanism of action. [26]

It is well established that ATQ has a long half life (70 to 84 hours), exert its effects on the parasite within minutes after drug treatment [27], can be administered via the oral rout and has an excellent safety profile and tolerability. The most common registered side effects are rash, fever, vomiting, diarrhea, abdominal pain and headache [28], and there is no registration of any side effects that obligate withdrawal of therapy[21]. The absence of severe side effects of this drug can be attributed to its selectivity as mentioned above.

Despite of these advantages; ATQ is associated with some limitations that affect its effectiveness.

Different approaches are employed to prolong the pharmacological activity, increase oral bioavailability and decrease inter-individual variability of ATQ. In general, the prodrug approach is one of polices that is used to enhance the pharmacokinetic behavior of drugs like ATQ. It includes the conjugation of the parent drug to a linker to produce a system that is able to release the parent drug once in the body. The next section shed the light on prodrugs as a powerful means in the drug development process.

1.1.2 Prodrugs

In the past few decades the pharmaceutical sciences have been subjected to considerable alterations [29] in terms of improving drug drawbacks related to pharmacokinetic (absorption, distribution, excretion, and metabolism) pharmaceutical and biological performance of existing drugs which may hinder drug development course [30].

Overcoming the undesirable physicochemical, biological and organoleptic properties of some existing drugs [29] can be achieved through the development of new chemical entities with desirable efficacy and safety. However, this is an expensive and time consuming process that needs a screening of thousands of molecules for biological activity [31] in addition to the rigorous rules and criteria that are applied today for developing new drugs [16]. So that, it becomes much more feasible to modify and improve the properties of already existing drugs through exploring the prodrug approach [31] in order to get red of their undesirable features and to increase their commercial life cycle and patentability [30].

Prodrugs are inactive forms of active drugs that are designed to exhibit pharmacological activity after an enzymatic or chemical reaction when they have been administered into the body [32]. Prodrug approach is a promising and well established strategy for the development of new entities that possess superior efficacy, selectivity and reduced toxicity over their parent compounds. Hence an optimized therapeutic outcome can be accomplished[31].

Approximately, 10% of all worldwide marketed medicines can be categorized as prodrugs, and in 2008 alone, 33% of all approved small-molecular-weight drugs were prodrugs [33], and this confirms the success of prodrug approach [30].

In general, prodrugs are designed to 1) improve aqueous solubility ,2) to enhance permeability through modifying lipophilicity, 3) to achieve site specific delivery and to increase GI absorption through targeting specific transporters and enzymes 4) to improve taste, odor and other pharmaceutical and pharmacokinetic properties[31].

The classic prodrug approach is focusing on changing physicochemical parameters. Recently, modern computational methods are used to design linkers for drugs, more details are provided in the following section.

1.1.2.1 Design of innovative prodrugs using modern computational methods

In a similar manner to that utilized for drug discovery, modern computational methods based on molecular orbital and molecular mechanics methods are explored for the design of innovative prodrugs for drugs containing hydroxyl, phenol, or amine groups. For example, mechanisms for several enzyme models that have been utilized to understand enzyme catalysis have been recently studied by Karaman's group and used for the design of some novel prodrug linkers [34]. The classic prodrug approach was focused on altering various physiochemical parameters, whereas the modern computational approach, considers using a design of linkers for
drugs having poor bioavailability, to provide the parent drugs in a controlled release manner, and thus improving their bioavailability. In addition, since the linkers used are relatively small molecules, it is expected that the prodrugs themselves might be with considerable biological effects before they interconvert to the parent drugs.

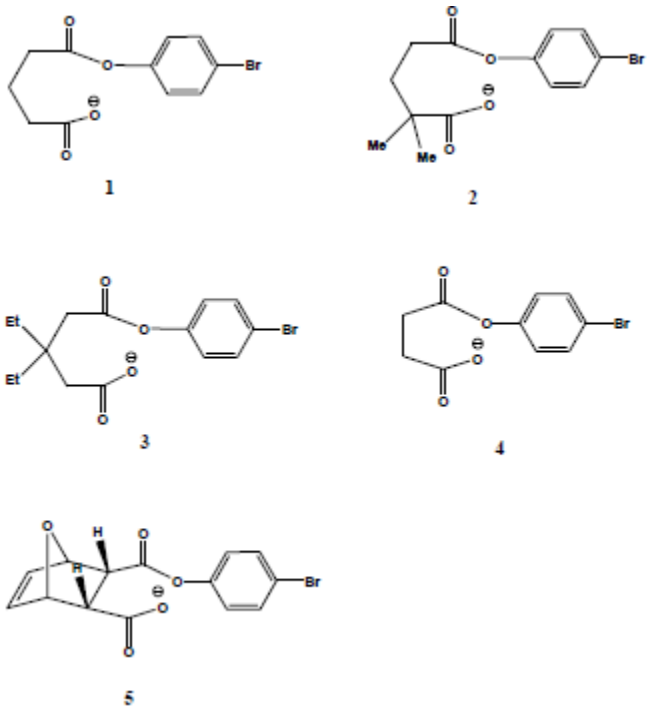
Using modern computational methods like DFT, molecular mechanics and ab initio methods, numerous enzyme models were explored for determining the factors playing dominant role in governing the reaction rate in such models. Among the enzyme models that have been studied are: (a) proton transfer between two oxygens and proton transfer between nitrogen and oxygen in Kirby's acetals [35] (b) intramolecular acid-catalyzed hydrolysis in maleamic acid amide derivatives [35] (c) proton transfer between two oxygens in rigid systems as investigated by Menger [36]; (d) acid-catalyzed lactonization of hydroxyl-acids as studied by Cohen [37] and Menger; and (e) SN2-based cyclization as studied by Brown [38], Bruice [39], and Mandolini [40].

The interconversion of a prodrug to the parent drug at the target site is a necessity for the prodrug approach to be successful [41]. The major obstacle facing the prodrug approach is the difficulty in predicting the bioconversion rates, and thus the pharmacological or toxicological effects of the prodrugs [42] [43].

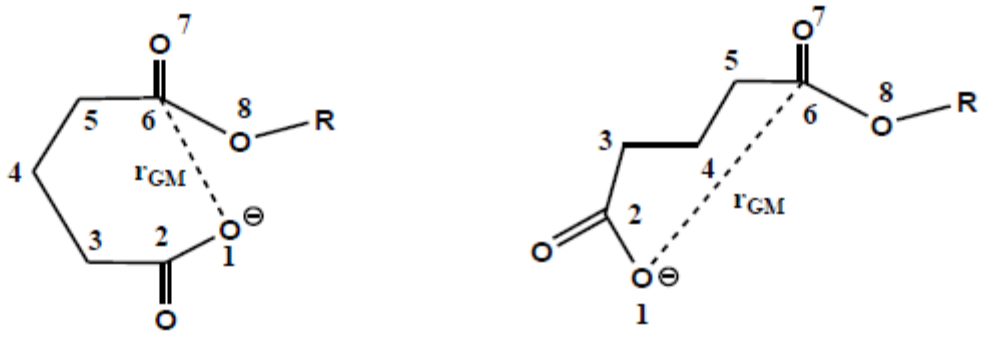
Karaman's studies on intramolecularity [43] have revealed that there is a necessity to further explore the reaction mechanism for determining the factors affecting the reaction rate. Unraveling the reaction mechanism would allow for better design of an efficient chemical device to be used as a prodrug linker that can be linked to a drug moiety which can chemically, and not enzymatically, cleaved to liberate the active drug in a programmable and controlled manner.

For ATQ, Bruice's model was employed to help in the design of ATQ prodrugs that have the expected characteristics.

Bruice's systems **1**–**5 scheme 1.1** indicate that the rate enhancement in the cyclization of di-carboxylic semi-esters **1**–**5** is solely the result of strain effects and not as a result of proximity orientation due to the 'reactive rotamer effect, scheme 1.2.



*Scheme 1.1: Chemical structures for di-carboxylic semi-esters **1-5**.*



R= *p*-bromophenyl or ATQ moiety.

*Scheme 1.2: Schematic representation of the reactants in the cyclization reactions of dicarboxylic semi-esters **1**-**5** and ATQ **ProD 1**-**ProD 5**. r_{GM} is the distance between the nucleophile (O1) and the electrophile (C6).*

In addition, it was found that the activation energy in systems **1**-**5** is dependent on the difference in the strain energies of the tetrahedral intermediates and the reactants, and there is no correlation between the cyclization rate and the distance between the nucleophile and the electrophile (r_{GM}). Therefore, the inter-conversion rates of atovaquone prodrugs to atovaquone can be programmed according to the nature of the prodrug linker [44].

1.2 Problem statement

Given the severity of Malaria, continual development of drug resistance and the undesirable safety profile of some existing medications, efforts were directed toward development of more effective, better tolerated medications with lower propensity to develop resistance, intended for the treatment of this endemic disease [8].

ATQ hold promise in malaria treatment, owing to its unique mechanism of action, effectiveness and safety. However, ATQ has poor oral bioavailability (<10% under fasted condition) and variable oral absorption , and this is due to its poor aqueous solubility (<0.2 μg/mL) that results from its lipophilic structure Log P= 5) [45]. Consequently, this results in low and variable plasma and intracellular levels of the drug which is an important determinant of therapeutic outcome [46]. It was demonstrated that low drug plasma

concentrations is a powerful means for the promotion of resistant parasites [47] eased morbidity and mortality among children [48]. Furthermore, ATQ is an expensive medication [49]. ATQ oral bioavailability can be increased either by fatty food intake [50], or administering larger amount of the drug to recompense for low oral absorption and to reach therapeutic plasma concentrations [20]. This practice is considered to be costly with expensive drugs like ATQ. Altogether, these procedures hinder the use of ATQ in poor developing countries in the time in which ATQ is considered to be the standard antimalarial [28].

Thus, the adoption of strategies to protect ATQ against resistance is an urgent need [16]. Accordingly, to achieve therapeutic success, ATQ solubility improvement strategies should be addressed; in order to meet the medical needs in malaria treatment.

1.3 Goals and Objectives

1.3.1 Research goals

In the view of this background and continuing Karaman's study on design and synthesis of ATQ prodrugs, this research was conducted to synthesize a previously designed ATQ prodrug, through linking ATQ to a di-carboxylic semi-ester linker, like succinic anhydride (Bruice's enzyme model), to produce a system that is more hydrophilic than its parental drug, and is able to release ATQ in a chemically driven controlled manner, once in the body.

Consequently, novel ATQ prodrug that serve in providing an alternative treatment option to the medical community that may help in addressing the critical need in malaria treatment will be introduced.

In accordance to the thesis in hands, ATQ prodrugs are expected to fulfill the following requirements: (1) enhanced water solubility; (2) improved oral bioavailability; (3) controlled release rate; (4) predicted plasma levels and (5) improved antiparasitic activity.

1.3.2 Specific Objectives

In this context, to achieve our research goal the following specific objectives have to be accomplished:

1- To extract ATQ from (Mepron®) GlaxoSmithKline, tablets (250 mg Atovaquone + 100 mg Proguanil Hydrochloride).

2- To synthesize ATQ succinate, by conjugating ATQ with succinic anhydride, through, simple one step chemical reaction.

3- To purify and characterize the proposed prodrug using several purification and characterization techniques.

4- To perform *in vitro* prodrug kinetic analysis in different pH solutions (pH 2.2, pH 5.2 and pH 7.4).

1.4. Research Questions

The research questions for the thesis in hand are:

1. Would it be possible to link ATQ to water soluble linker via chemical synthesis?
2. Will the prodrug have superior water solubility over ATQ?
3. Will the prodrug be able to release ATQ in a controlled manner?

Literature Review

Chapter Two

Literature Review

Accumulating evidence suggests that ATQ low solubility and hence low oral bioavailability and variable plasma concentration limits ATQ inherent efficacy,[45], [3] [51] ,[52].

In addition, several studies reported that sufficiently high ATQ plasma levels should be achieved to obtain the desired therapeutic response [45]. It was demonstrated in clinical study with a conventional tablet formulation, that the therapeutic response of ATQ against Pneumocystis carinii Pneumonia is reliant on plasma steady-state levels of the drug [20]. Moreover, Chung, Ferreira et al [53] mentioned that resistance to ATQ was reported which is believed to be a result of low and variable plasma levels that results from variable oral absorption. This conclusion could be supported by the fact that inconsistent drug plasma levels will provide the parasite with the opportunity to form resistance against drugs. All of the mentioned complications result from ATQ lipohilic properties.

Despite of this, there is a general agreement that this problem is solvable [45], [3], [51], [52]. So, different techniques were adopted in order to minimize solubility and bioavailability problems of ATQ.

2.1 Approaches adopted to enhance ATQ aqueous solubility

Atovaquone was firstly commercialized as tablets (Mepron®), from which complete oral bioavailability can't be achieved. About 21% absolute bioavailability of Mepron® tablets was obtained in HIV seropositive volunteers in the fed state [54]. Therefore, Different groups focused on improving ATQ solubility via several approaches, for example: Comley, Yeates et al [45], Karaman and Hallak [3], Hughes, Dorenbaum et al [51], , Dixon, Pozniak et al. [52], etc…

Most work is directed toward improving ATQ formulation to enhance bioavailability.

Strategies that are employed focus on an increment of the specific surface area of atovaquone particles and/or its solubility in adequate solvents or micelles to facilitate its dispersion in aqueous media.

For example, Cotton, 1995 [55] developed micronized ATQ suspension and compared it with ATQ tablets, and he found that micronized ATQ suspension achieved 2 fold increase

in drug bioavailability compared to that with tablet formulation of the same dose. These findings were reported in both the fed and fasted state [52]. It was indicated earlier that ATQ absorption can be increased when administered with food [52] for both tablet and suspension formulations. It was stated that a1.4-fold increase in ATQ absorption was obtained in the fed state, compared to that achieved in the fasting state [50]; and this amount can be higher depending on the fat content of the meal [54].

Additional methods to improve atovaquone oral bioavailability have been exploited, including the development of nanosuspensions [56], self micro-emulsifying drug delivery systems (SMEDDS) [57][64], liposomes [58] or polymer nanocapsules [59].

In this context, Dearn and coworkers revealed in male volunteers that the administration of microfluidized suspensions of 0.1-2 μm average leads to 2.6-fold increase in oral relative bioavailability than when a typical suspensions (of about 3 μm) was used[56].

In another interesting study, Sek and collaborators examined the influence of a number of surfactants; selfmicroemulsifying drug delivery systems on the oral bioavailability of lipid based formulations of atovaquone [57].

No differences were observed in beagle dogs when comparing two different (SMEDDS)

On the contrary, the relative oral bioavailability in dogs of atovaquone was about 3-fold higher when incorporated in these self micro-emulsifying drug delivery systems (SMEDDS) than when formulated as aqueous suspension.

Another fascinating option is the association of this drug with bio adhesive nanoparticles. In this case the strategy combines an increase of the specific surface area of the drug delivery system with the ability of these nanoparticles to develop adhesive interactions within the gut mucosa, which may assist in the formation of a concentration gradient between the dosage form and the gut mucosa, thus enhancing absorption potential.[60].

Despite of being attractive options, the mentioned strategies add other steps to the process, in addition to the increased cost. Another option is to modify the structure of the drug in such a way to enhance poor bioavailability by increasing aqueous solubility of the drug [20].

Hage et al [61], reveals the synthesis and antimalarial activity of new atovaquone derivatives, which was substituted at the 3-hydroxy group by ester and ether functions. The compounds were assessed in vitro for their activity against the growth of Plasmodium falciparum,. It was demonstrated that all the compounds exhibited potent activity, with IC50 values in the range of 1.25-50nM, comparable to those of atovaquone and much higher than chloroquine or quinine [20]**.**

On the other hand, Comley [45] and Karaman [3] also shed the light on modifying ATQ at the structural level. However, their idea was to link ATQ to a water soluble moiety to develop prodrugs rather than developing ATQ derivatives to enhance aqueous solubility.

Comley developed the carbamate prodrug of ATQ 17C91 and compared plasma levels of 17C91 with the micronized ATQ suspension in a severe combined immunodeficient mouse model of Pneumocystis carinii pneumonia (PCP). Comley found that there is a 3 fold increase in plasma levels compared to that with micronized ATQ suspension, which indicates that ATQ prodrugs are superior over both tablets and micronized suspensions.

Comley prodrug 17C91 system has proved its efficacy and this confirms our expectations for improvement ATQ characteristics using the linker approach. However, 17C91 releases ATQ very rapidly ($t_{1/2}$ 3 minutes at pH 7.4), in pH dependent manner without any control on drug releasing rate [45] .

Another group adopted the development of ATQ prodrugs [20], in order to improve ATQ aqueous solubility. The new compound is synthesized by condensation of atovaquone with 5-methyl-4-chloromethyl dioxalone (III) in a suitable solvent system and optionally followed by distillation and re-crystallization to obtain Atovaquone prodrug compound of formula 3-(5-methyl-2-oxo-l, 3-dioxol-4-yl) methyloxy-2- trans- [(4-chloro phenyl) cyclohexyl][l ,4] naphthoquinone [20].

The main advantage of Karaman's proposed prodrugs lies in their ability to release ATQ via chemical cleavage in a controlled manner depending on the nature of the linker. This ensures a sufficient ATQ plasma levels that can be maintained long enough due to controlled ATQ releasing rate, which subsequently increases the probability of therapeutic success [3].

It is worth noting that enzymes have a significant role in prodrugs transformation into the parent compounds. Many of the marketed prodrugs realize on this hydrolytic mechanism to elaborate the parent drug, by catalysis of peptidases, phosphatases, carboxylesterases or esterases [40]. However, this pathway is associated with obstacles that hinder its usefulness. For instance, the incomplete absorption obtained with several hydrolyze-enzymes-activated prodrugs of antibiotics, and angiotensin-converting enzyme inhibitors, such as enalaprilate, leads to about 50% bioavailability, because of their premature hydrolysis by esterases during the absorption process[40]. Additional important issue is the bioactivation of the prodrug by cytochrome P450 enzymes. The latter are a class of enzymes that accounts for about 75% of all enzymatic metabolisms of drugs, including several prodrugs. There is increasing evidence that genetic polymorphisms of prodrug-

activating cytochrome P450 enzymes significantly contribute to the variability in prodrug activation and thus to efficacy and safety of drugs utilizing this pathway [62].

The prodrugs chemical approach involving enzyme catalysis is perhaps the most vulnerable and unpredicted approach, because there are many intrinsic and extrinsic factors that can affect the bioconversion mechanisms. For example, the activity of many prodrug activating enzymes may be flocculated due to genetic polymorphisms, age-related physiological changes or drug interactions, leading to adverse pharmacokinetic, pharmacodynamic and clinical effects. In addition, there are wide interspecies variations in both the expression and function of most of the enzyme systems activating prodrugs which could lead to serious challenges in the preclinical optimization phase[40].

Herein, lies the significance of the of Karaman's prodrugs, that are converted chemically into the parent drug, and so the challenges associated with the enzymatic hydrolysis are avoided like the problems of high and slow metabolizers and the consequences of these effects on drug effectiveness and toxicity, respectively. Likewise, the programmed release allows for sufficient ATQ levels that are maintained long enough in the plasma, in a consequence a once daily dose is allowed which leads to improved patient compliance.

Materials and Methods

Chapter Three

Materials and Methods

This chapter consists of two major parts. The first one is the Synthetic chemistry part which concerns with the reactions, reagents, solvents and materials used. And the second is the Kinetic studies part which describes the specific preparations and analysis used to investigate ATQ prodrugs hydrolysis in different pH solutions using the HPLC instrument.

3.1 Part one

3.1. 1 Chemicals and Instrumentation.

3.1.1.1 Reagents

Succinic anhydride, sodium hydride (NaH), thin layer chromatography (TLC), silica gel, NaCl, NaOH, anhydrous magnesium sulfate MgSO4, are all purchased from Sigma Aldrich. Atovaquone was obtained from commercial Malarone tablets (250 mg ATQ and 100mg proguanil) GlaxoSmithKline Inc.

3.1.1.2 Solvents

Dioxane, acetonitrile (ACN), hexane, ethyl acetate, dimethylformamide (DMF), chloroform ($CHCl_3$), dimethyl sulfoxide (DMSO), NaOH, methanol (MeOH), and diethyl ether, tetrahydrofurane (THF) and distilled water were used directly from the bottles, and all were purchased from Sigma Aldrich.

3.1.1.3 Instrumentation and substance identification.

Chemical hazards fuming hood, vacuum pumps, hotplates, available at Karaman's Lab in the Faculty of Pharmacy, Al-Quds University. FTIR, pH meter, HPLC and rotary evaporator are available at Al-Quds University. ^{1}H-NMR and LC/MS were taken at the Hebrew University (the names of the companies are provided below).

All of these instruments were employed to characterize, and to describe the physicochemical properties of our compound of interest.

3.1.1.3.1 Melting point determination by capillary method.

Capillary method is commonly used in chemistry labs to determine the melting points of solid substances. This technique is easy and requires a small amount of the material. It is performed by introducing a small amount of the solid into a one end sealed capillary tube, which is then fixed into a thermometer, then dipped into an oil bath. Heating of the oil bath should be done slowly and gently, to ensure uniform heating of the sample and the thermometer. Then, the temperature range over which the sample starts to melt is recorded to be as the melting point of the material.

3.1.1.3.2 Log P

Log P was determined using Chem draw Ultra 7 program [63].

3.1.13.3 High performance Liquid Chromatography HPLC.

HPLC from (Waters 2695: Israel), (Shimadzu corp. Japan), and waters Micromass® Masslynx ™ detector with Photo diode array (PDA) (Waters 2996: Israel). Data acquisition and control were carried out using Empower ™ software (Waters: Israel).
Analysis was done using C_8, 4.6 mm x125 mm, 5 μm particle size, protected by XBridge® C8 guard column. Micro filters 0.45μm porosity were used (Acrodisc® GHP, Waters). The C-8 (1 gm) cartridges 6cc single use for laboratory use, were purchased form Waters Company (Milford, MA, USA).

3.1.1.3.4 pH meter

pH values were recorded on pH meter model HM-30G: TOA electronics ™ was used to measure pH values for prepared buffers.

3.1.1.3.5 FT-IR

All infrared spectra (FTIR) were obtained from KBr (potassium bromide) matrix (4000–400 cm^{-1}) using a PerkinElmer Precisely, Spectrum 100, FT-IR spectrometer.

3.1.1.3.6 Nuclear magnetic resonance spectroscopy (^{1}H -NMR)

All NMR spectra were gained by using the 400 MHz Varian NMR spectrometer. One of the experimental samples was run in DMSO-d6 and the other in $CDCl_3$. 1H NMR experiments are stated in parts per million (ppm) downfield of TMS.

The following symbols used for 1H NMR peak investigation: chemical shift (δ ppm), multiplicity (s =singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration and coupling constant (Hz). All NMR data were analyzed by MestReNova Software.

3.1.1.3.7 Liquid Chromatography- Mass Spectroscopy (LC-MS)

HPLC–MS/MS Shimadzu prominence high performance liquid chromatography system (Shimadzu corp. Japan) was employed to record LC/MS measurements, at the Hebrew University.

3.1.2 Chemical synthesis and purification

This section describes the chemical reactions of linking ATQ to succinic anhydride to yield ATQ succinate prodrug.

3.1.2.1 ATQ extraction from Malarone tablets

ATQ was isolated as follows. Twelve Malarone tablets (3000 mg, 8.19 mmol) were crushed using a mortar and pestle. The resulting powder that consists of ATQ, proguanil and other ingredients was washed with 9 ml chloroform ($CHCl_3$) added on 3 portions in order to dissolve ATQ while other ingredients are insoluble, then filtration was done and the container was washed with $CHCl_3$ to remove any residual powder. ATQ was further extracted using $CHCl_3$ and a saturated NaCl solution (NaCl was added to exert salting out effect).The organic layer was separated and the aqueous layer was further extracted with $CHCl_3$, then the combined organic layers were dried over anhydrous $MgSO_4$ to make sure the complete removal of H_2O, then $CHCl_3$ was evaporated. Then yellow ATQ powder was collected and further purified by recrystallization using ACN (sufficient quantity). After recrystallization filtration was done to obtain precipitated ATQ, then the precipitate was dried and collected. Thus dry and pure ATQ was ready to be used. Afterwards, the product was tested by melting point (M.P), FT-IR, ^{1}HNMR and LC/MS.

3.1.2.2 ATQ Succinic anhydride conjugation reaction

This was prepared as follows. An oven dried round bottom flask was charged with 30mL THF, then 500 mg (1.363 mmol) of ATQ was added to the pot followed by adding 3 equivalents of NaH (4.089 mmol). The addition of NaH was performed at 10 ^{o}c that was accomplished by placing the RBF on an ice bath and the temperature of the bath was controlled with the aid of a thermometer. A magnetic stirrer was placed in the RBF. After 15 minutes, a 136 mg (1.363 mmol) of succinic anhydride was added to the reaction pot at 10^{o}c. The flask was air-tightened and closed with a flexible rubber stopper. The reaction was left over night at room temp for 3 days and monitored by TLC to ensure reaction completion using ethyl acetate and hexane (1:5) system as an eluent.

3.1.2.3 Purification of ATQ succinate prodrug using column chromatography

ATQ succinate prodrug was purified as follows.

The mixture was purified using conventional column chromatography. The column was prepared conventionally using silica gel that was purchased from Sigma Aldrich; the starting eluent is 10% ethyl acetate and 90% hexane. The polarity of the eluent was increased gradually until the compound is eluted out using 15% ethyl acetate and 85% hexane. TLC was performed continually as the fractions were obtained, to ensure product elution.

The fractions were collected and the solvent was evaporated by the rotary evaporator, then the resulting pale yellow prodrug was dried to yield 30% product. Then a full characterization of the prodrug was done by melting point, FT-IR, 1HNMR and LC/MS.

3.2 Part Two

3.2.1 Kinetic studies on the stability of ATQ succinate prodrug at different buffer conditions

3.2.1.1 In vitro conversion time evaluation

In vitro conversion time into the parent drug in aqueous media was evaluated.This was accomplished by evaluating chemical hydrolysis of the prodrug at pH 2.2, pH 5.5 and pH 7.4 solutions.

In order to determine the hydrolytic rate of the prodrug at different pH conditions; a specific preparations were performed as follows.

a) **Buffer preparation:** 7 gr. KH_2PO_4 was dissolved in 1L water, this yielded a solution that has a pH of 4.2 then pH was adjusted by either NaOH or 1N HCl to get the desired pH buffer.

b) **Stock solutions:** 1000 ppm stock solution of each ATQ and ATQ succinate prodrug was prepared by dissolving 25mg ATQ and ATQ prodrug in 25 ml DMF using a volumetric flask.

c) **Dilution:** A 500 ppm of ATQ and ATQ prodrug solutions were prepared by transferring 5ml of 1000 ppm stock solutions of each ATQ and ATQ prodrug prepared in (b) to a 10 ml volumetric flask and diluting it using DMF and the corresponding buffer.

Note: Because both ATQ and ATQ succinate prodrug have poor solubility in the three buffers, dilution was made by adding 5ml DMF and the volume was completed with each corresponding buffer to get clear solutions.

3.2.1.2 HPLC analysis

Injections into HPLC apparatus was made at (zero time,1,2 ,3,4,5 ,24 hrs after sample preparation then after one weak and so on, till 50% prodrug degradation was took place.

3.2.1.2.1 Chromatographic conditions

It is significant to mention that different attempts were made to optimize the mobile phase composition, pH, detection wave length, and the column used for the separation process. Our trials continued until the best possible chromatograms were obtained. The conditions that are tried are shown in the tables below.

As indicated in table (3.2), acetic acid was used to achieve acidic medium, (low pH of the m.ph) as an attempt to maintain the drug and the prodrug in unionized form. It is known that a buffered mobile phase is used for ionized compounds in order to maintain them in a specific form, so a reliable retention time is obtained in all runs. However, chromatograms show that ATQ prodrug is degraded when this mobile phase composition was used. Thus, the conditions were re-modified and the use of acetic acid was omitted, tables (3.3) and (3.4). This leads to the appearance of ATQ prodrug peak without any degradation. However, the retention time of ATQ is relatively high (20 minutes) when these conditions were used. So, the elution was re-modified to an isocratic one using ACN: H2O(90:10), in addition, C8 column was used instead of C18 column (ATQ and ATQ prodrug stuck to the column due to their lipophlic structure) which allows both of the drug and the prodrug to elute earlier (less than 5 minutes) with a good separations , tables (3.5) and (3.6).

The appropriate wave length λ was found to be 280 nm in all conditions. 280nm was chosen instead of 303nm, because at 280nm both the drug and the prodrug show the finest absorbance, while when 303 nm was used the prodrug peak was undetected.

Then the disappearance of the prodrug with time was followed up. Concentration versus time was plotted and hydrolysis rate at different buffers was estimated.

Table 3.1: The initial HPLC conditions that are used.

Condition I	**Stationary phase**	**Mobile phase**	**Flow Rate**	**Run Time**	**λ**	**Injection volume**
	C_{18}, 125 mm	Gradient elution 1	1ml/min	20 mins	303 nm	10µl

*Table 3.2: Gradient elution 1, in which B is ACN and **C** is 0.5%CH_3CO_2H in water.*

Number	**Time (min)**	**Flow (ml/min)**	**% B**	**%C**
1		**1**	**30**	**70**
2	**3**	**1**	**30**	**70**
3	**8**	**1**	**90**	**10**
4	**12**	**1**	**90**	**10**
5	**13**	**1**	**30**	**70**

Table 3.3: the modified chromatographic conditions.

	Stationary phase	Mobile phase	Flow Rate	Run Time	λ	Injection volume
Condition 2	C_{18}, 125 mm	Gradient elution 2	1ml/min	20 mins	303 nm	10µl

*Table 3.4: Gradient elution 2, in which **B is** ACN, **C is** water.*

Number	Time (min)	Flow (ml/min)	% B	%C
1		**1**	**30**	**70**
2	**3**	**1**	**30**	**70**
3	**8**	**1**	**90**	**10**
4	**12**	**1**	**90**	**10**
5	**13**	**1**	**30**	**70**

Table 3.5: The optimized HPLC conditions.

Condition 3	**Stationary phase**	**Mobile phase**	**Flow Rate**	**Run Time**	**λ**	**Injection volume**
	C_8,5µm4.6x125nm	Isocratic flow 90:10 ACN: H2O	1.5ml/ min	5 mins	280nm	10µl

Results and Discussion

Chapter 4

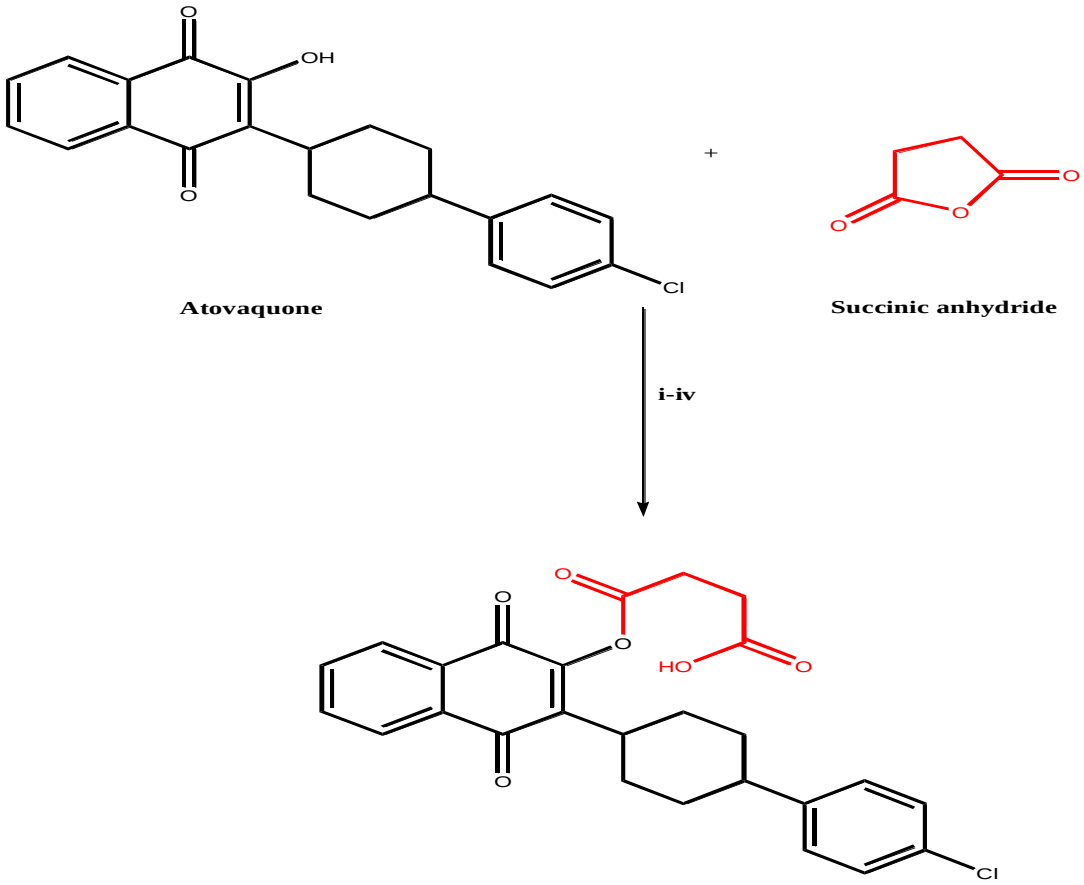
Results and discussion

ATQ Succinate prodrug (ProD1) is successfully obtained, and all the gained data are consistent with the proposed compound. A full description and analysis of the results are provided below.

4.1 Chemical synthesis

After ATQ extraction, a complete characterization of the obtained yellow powder was done by melting point, FT-IR, and H-NMR. M.p= 224^{o}C. 3397 phenol vibration, 3086 aromatic C-H vibration, 1647-1659 c=o stretching vibration, 727 aromatic chloride. H1-NMR (DMSO-d6, 400 MHz):δ 8.13(d,1H), 8.07(d, 1H), 7.76(t, 1H), 7.68 (t, 1H), 7.48 (s, 1H), 7.28 (d, 2H), 7.18 (d, 2H), 3.16 (t, 1H), 2.63 (t, 1H), 2.2 (m, 2H), 1.96 (d, 2H), 1.75 (d, 2H), 1.58 (q, 2H).

The scheme shown below describes the chemical reaction of ATQ and succinic anhydride (linker) to yield 30% of our proposed ATQ prodrug. M.p *˜280^{o}C.* , IR (KBr/ν_{max} cm^{-1}) , 3379 OH STRECH, 1750 aliphatic C=O stretch, 1647-1659 c=o stretching vibration, 727 aromatic chloride , 1HNMR (400Hz, DMSO-d6) δ 7.99(dd,2H), 7.84(dt,1H),7.77(dt,1H), 7.33(s,4H),3.31(s,3H),3.07 (t,1H), 2.56(t, 1H), 2.22(q,2H),2.12(d, 2H),1.65(d,2H), 1.56 (q,2H), 1.49 (s, 1H), *m*/*z* 467 (M+1).

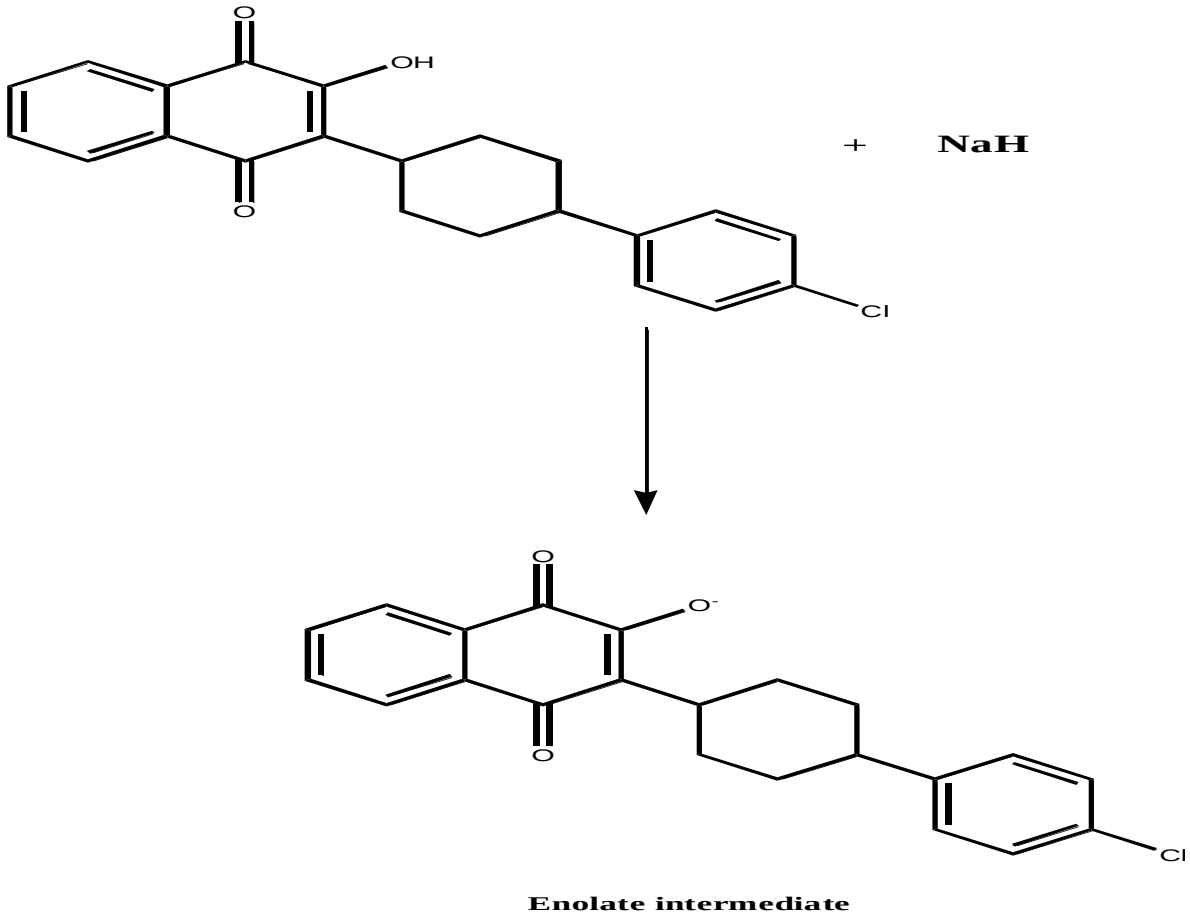


Scheme 4.1: shows the conjugation reaction of ATQ and succinic anhydride. Conditions and reagents :(i): THF, (ii): NaH, (iii): 10 °c then R.T, (iv): 3 days.

The scheme demonstrates a conjugation reaction in which a nucleophilic attack (SN_2 reaction) takes place between the OH group of ATQ and the electrophile center of succinic anhydride. This attack results in ring opening of succinic anhydride and the formation of an ester bond in the given prodrug, which is supposed to be cleaved once in the body depending on the pH of the medium.

To ensure the progress of the reaction in the suggested mechanism, specific reagents were used at certain conditions. For example, THF is a commonly used basic solvent in chemical reactions; it has a dielectric constant of 7.58. The basic conditions created by the solvent may encourage the deprotonation of the hydroxyl group of ATQ, hence increasing its nucleophlicity.

Moreover, Sodium hydride is a frequently used base to deprotonate alcohols, phenols, amides, ketones, esters and other functional groups for the promotion of their nucleophilic substitution. [64].The reaction was carried out at 10 c, because the enolate intermediate that forms after NaH addition is stable at low temperature rather than high Celsius degrees, Scheme (4.2).



Scheme 4.2: demonstrates the formation of enolate intermediate after adding NaH to ATQ.

The intermediate shown in the figure is given the time to be formed after mixing ATQ with NaH, and this is the reason of adding succinic anhydride after 15 minutes to the reaction pot.

TLC was conducted to monitor reaction progress using an eluent system that is composed of ethyl acetate and`` hexane in a ratio of 1:5. This ratio was specifically employed after various attempts to choose the best solvent system. For example 1:5, 2:5 methanol: hexane and 2:5 ethyl acetate: hexane were tried until the best $R._f$ values was obtained for the 1:5 ethyl acetate: hexane.

After the reaction was completed, a purification of the prodrug was done using column chromatography. Then a purity and identity confirmation assessments were conducted. A demonstration of the results of these examinations is provided in the following sections.

4.1.1 ATQ succinate prodrug identification

4.1.1.1 Log P

Log P is an important physical property that demonstrates the extent of aqueous solubility of certain compound. Log P can be measured by estimating the ratio of the concentration of the compound in an organic solvent (usually 1-octanol) to the concentration in aqueous solvent (water). High ratio means high organic solubility and vice versa [65]. Log P of ATQ ProD1was estimated using ChemDraw Ultra 7, to be 3.57, while that of ATQ is 5.1 [66].

4.1.1.2 Melting point of ATQ succinate prodrug

As a preliminary analysis of the material that was obtained from the reaction and purified by column chromatography, melting point was performed for both ATQ and the proposed prodrug by capillary method as a technique that is commonly employed in chemistry labs to analyze solid chemical materials and provides an initial information about the identity and the purity of different compounds.

It was given that the melting point of ATQ was 224^{o}C, while that for ATQ prodrug was 280 ^{o}C which is different from that of ATQ. This gives a preliminary indication that the compound is not ATQ.

4.1.1.3 FT-IR

4.1.1.3.1 FT-IR Spectra of ATQ:

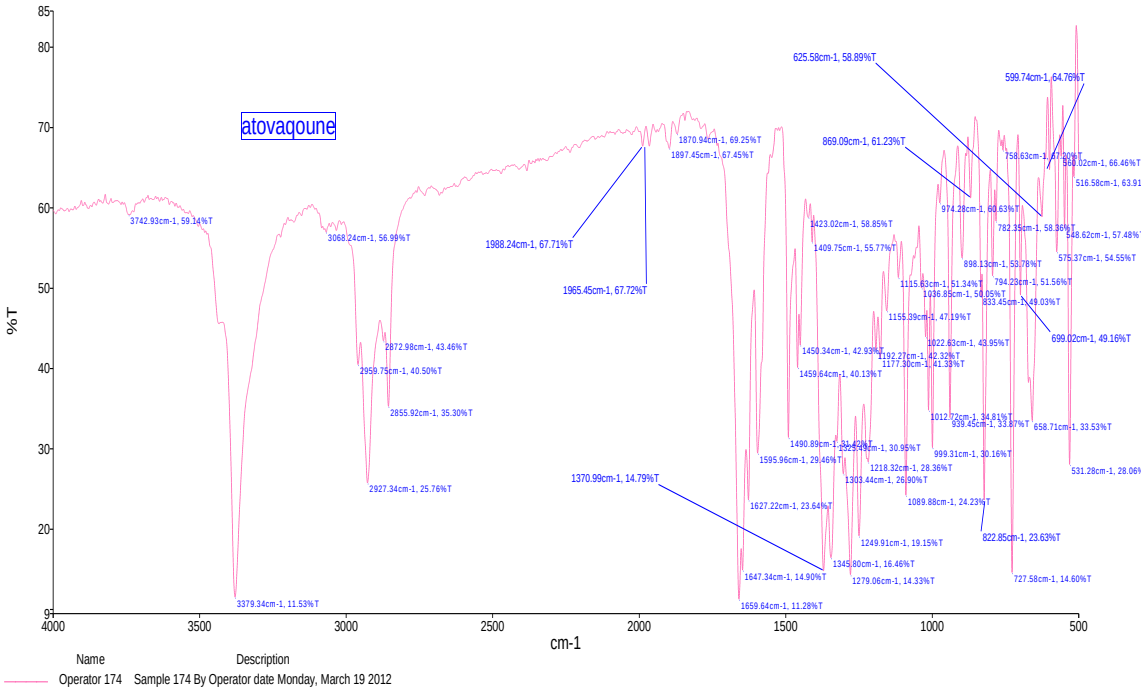


Figure 4.1: FT-IR spectrum of ATQ.

The spectrum analysis is as follows:

3397 phenol vibration, 3086 aromatic C-H vibration, 1647-1659 c=o stretching vibration, 727 aromatic chloride.

4.1.1.3.2 FT-IR Spectrum of ATQ ProD1:

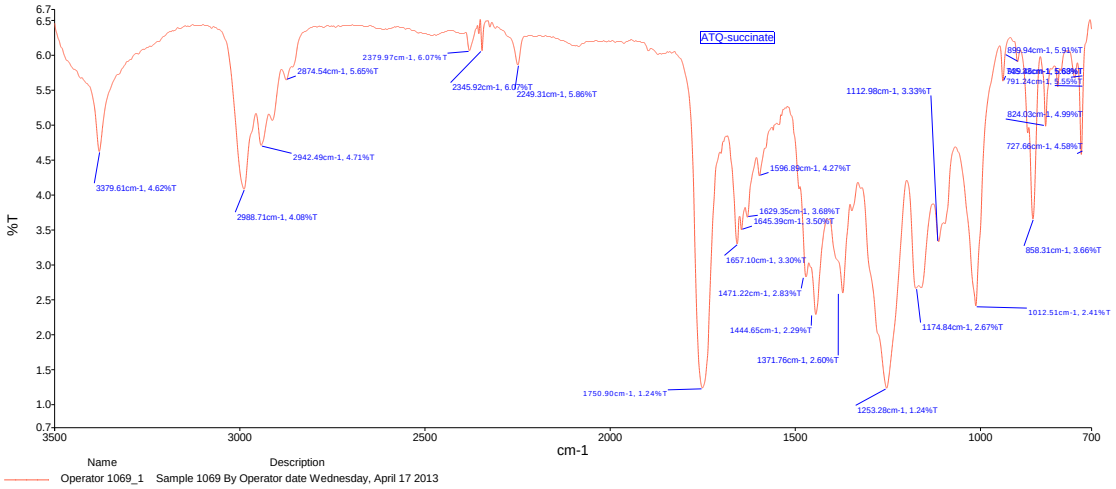


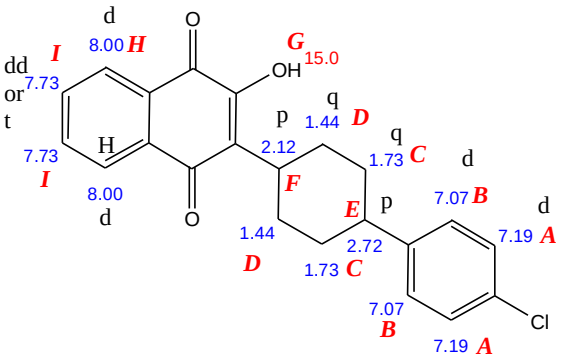
Figure 4.2: FT-IR Spectrum of ATQProD1.

3379 OH stretch, 1750 aliphatic C=O stretch, 1647-1659 c=o stretching vibration, 727 aromatic chloride.

It can be concluded from the spectrum of the prodrug that an aliphatic stretching of C=O group at 1750 is present, which is most likely indicate the presence of the carbonyl group of the succinate linker, in addition to the presence of signals of common groups in both the drug and the prodrug.

4.1.1.4 ^{1}H-NMR

4.2.3.1^{1}H-NMR of ATQ:



19 Hs

19Hs, M.W=366g/mol

Figure 4.3: Chemical structure of ATQ.

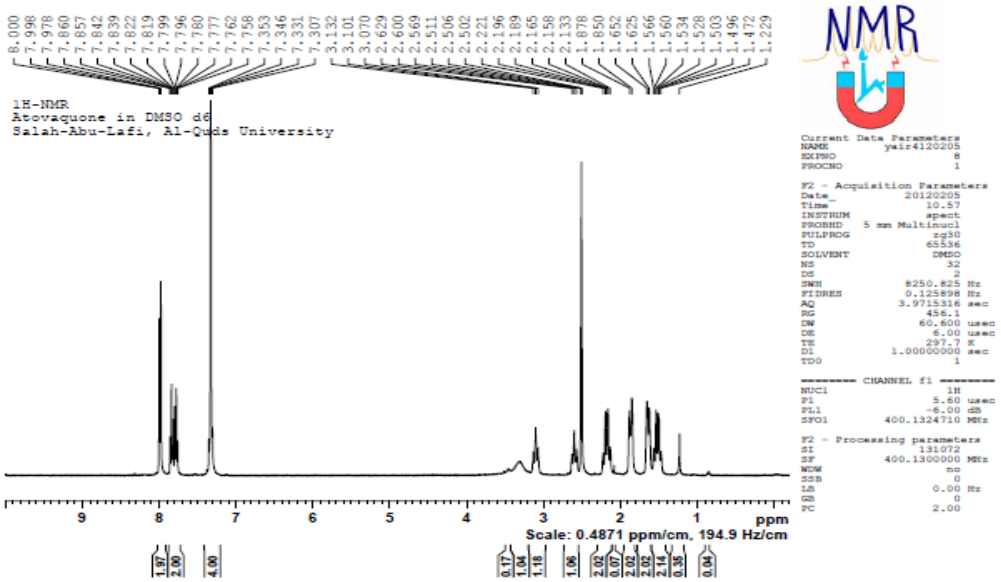


Figure 4.4: [1]H-NMR of ATQ; whole spectrum.

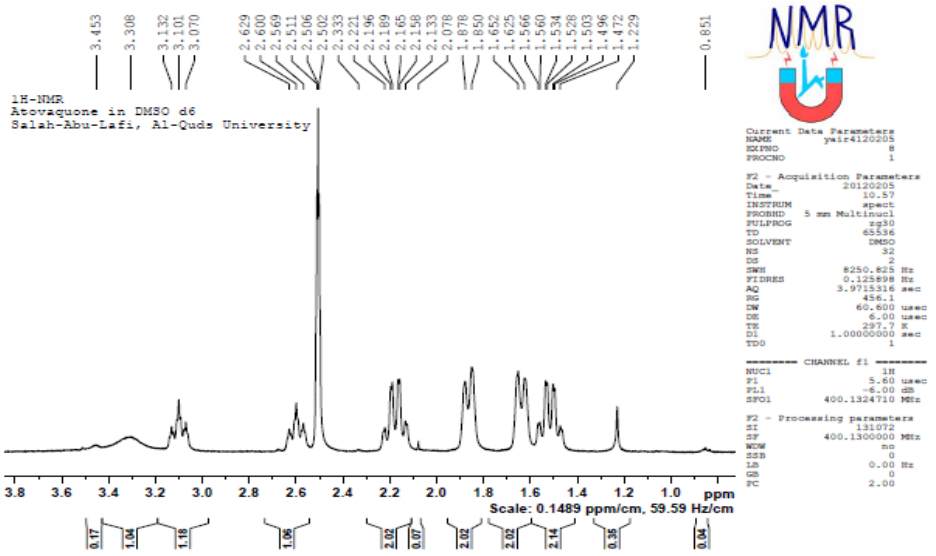


Figure 4.5: *[1]H-NMR of ATQ; aliphatic part.*

Table 4.1: Assignment of H-NMR signals of the aliphatic part of ATQ.

	C and C'	*D and D'*	*E*	*F*	*Total # of Hs*
Chemical shift	1.51 C' 1.62	2.2 D' 1.88	2.6	3.1	
Integration *1H ⟶ 1.03*	C : 2.14/1.03=2.07 ~2 Hs C': 2.02/1.03=1.96 ~2 Hs	D: 2.02/1.03=1.96 ~2 Hs D' : 2.02/1.03=1.96 ~2 Hs	1.06/1.03= 1.02 ~1H	1.18/1.03= 1.1 ~1H	**10 Hs**
Splitting	Q Or dt	Q Or dt	P	p	

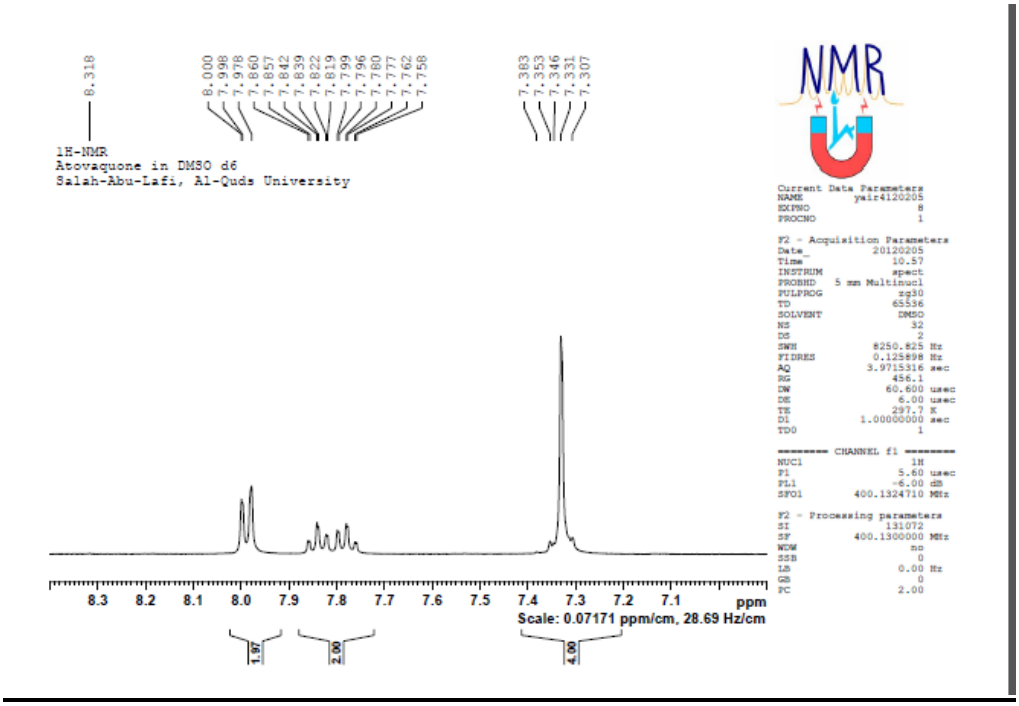


Figure 4.6: [1]H-NMR of ATQ; aromatic part.

Table 4.2: Assignment of H-NMR signals of the aromatic part of ATQ.

	A	*B*	*H*	*I*	*Total Hs*
Chemical shift	7.33	7.33	7.99	7.8	
Integration *(Σ areas=19.6)* *19.6 → 19H* *?? → 1H* *1H=1.03*	4/1.03= 3.9 ~4 Hs For A and B	Included in A peak	2/1.03= 1.9 ~2 Hs	1.97/1.03= 1.9 ~2 Hs	***8Hs***
Splitting	d	d	d	d	

4.1.1.4.2 H-NMR of ATQ succinate prodrug

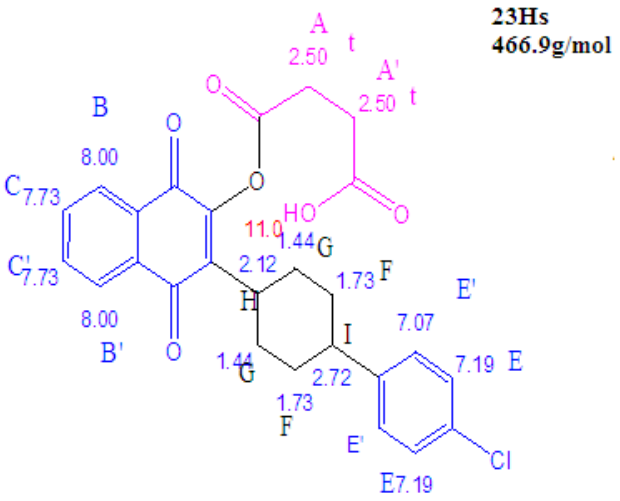


Figure 4.7: Chemical structure of ATQ succinate prodrug (ATQ ProD1).

The H-NMR spectrum of ATQ succinate prodrug is shown the figure below.

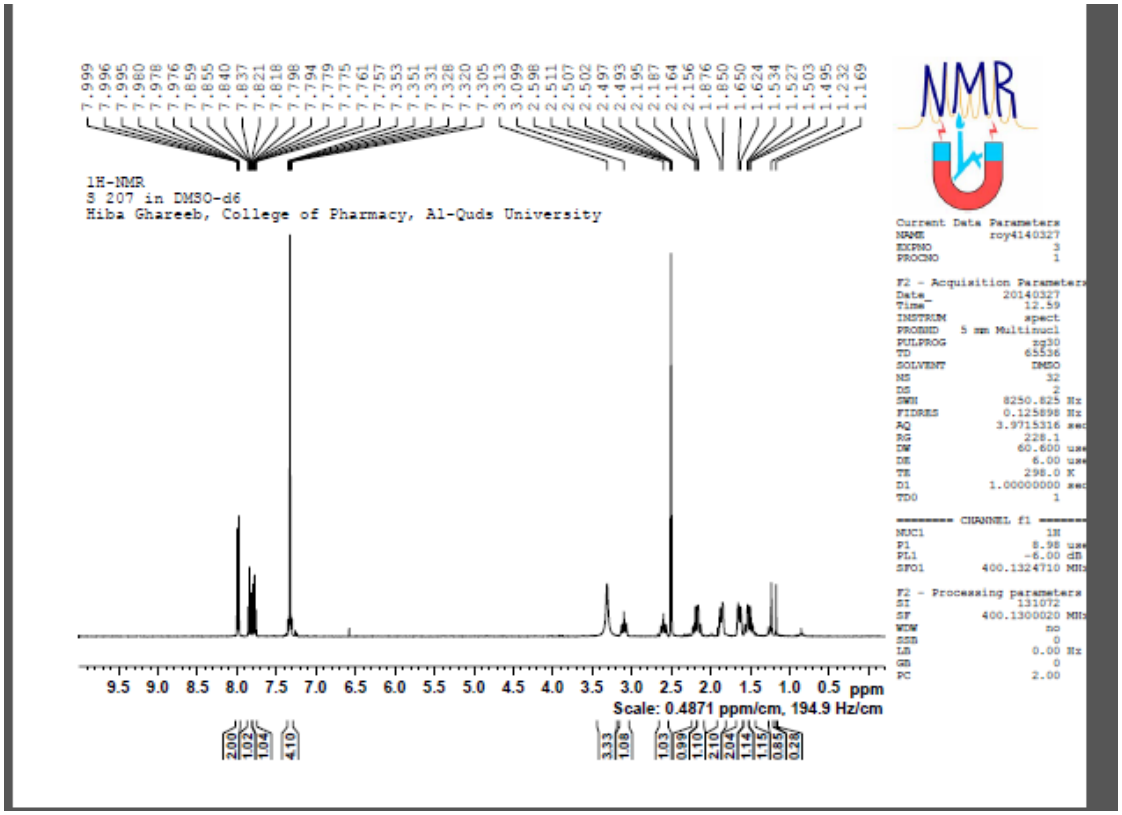


Figure 4.8: ATQ ProD1 [1] HNMR spectrum.

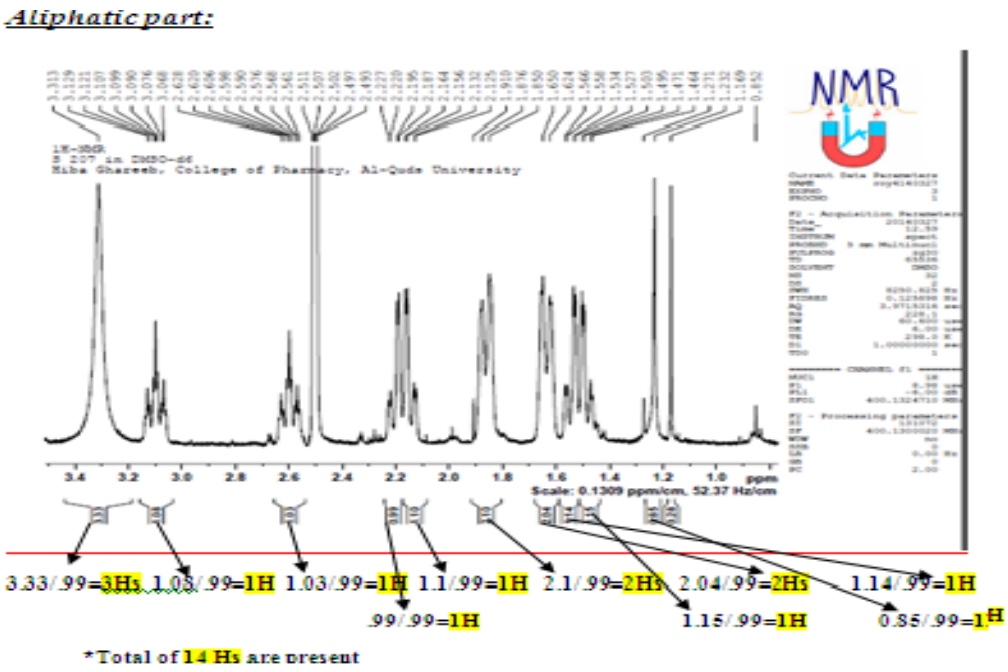


Figure 4.9:[1] H-NMR of ATQ ProD1; the aliphatic part.

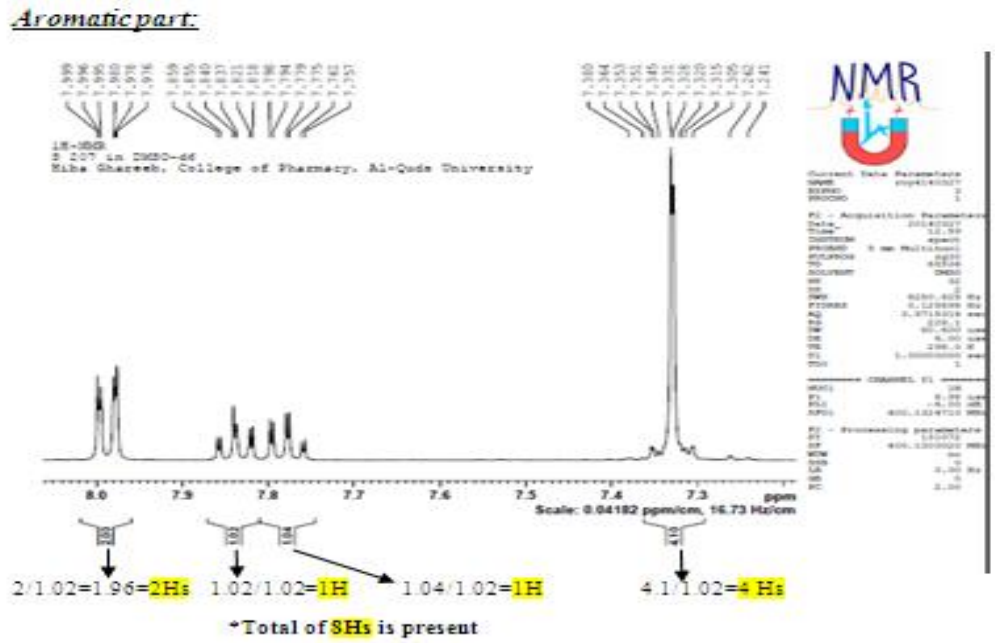


Figure 4.10:[1] H-NMR of ATQ ProD1; the aromatic part.

1HNMR (400Hz, DMSO-d6) δ 7.99(dd,2H), 7.84(dt,1H),7.77(dtt,1H), 7.33(s,4H),3.31(s,3H),3.07 (t,1H), 2.56(t, 1H), 2.22(q,2H),2.12(d, 2H),1.65(d,2H), 1.56 (q,2H), 1.49 (s, 1H).

As illustrated in the spectrum of ATQ Succinate, the pattern of signals is almost consistent with the structure of the proposed compound. The signals that appear at the aromatic region indicate the aromatic protons of the aromatic rings in the compound. the singlet signal that appear at 7.33 is integrated to 4 protons of ring E, figure (4.7), while signals that appear at 7.99, 7.84 and 7.77 splitted as dd, dt and dt and integrated into 2H, 1H and1 H each, additionally they appear at the aromatic region as mentioned earlier and therefore they were assigned as the protons of ring B, figure (4.7).

The assignment of the aliphatic part of the compound is a little bit more complicated, as with the available data it is difficult to make an absolute assignment of these signals.

The protons of the cyclohexane ring and their integrations are indicated with arrows as shown in figure (4.9) and (4, 10). The triplet signal that appears at 2.6 is assigned as being most likely the protons of the succinate linker of the prodrug; this almost indicates that the linker was successfully joined to ATQ.

4.1.1.5 LC/MS.

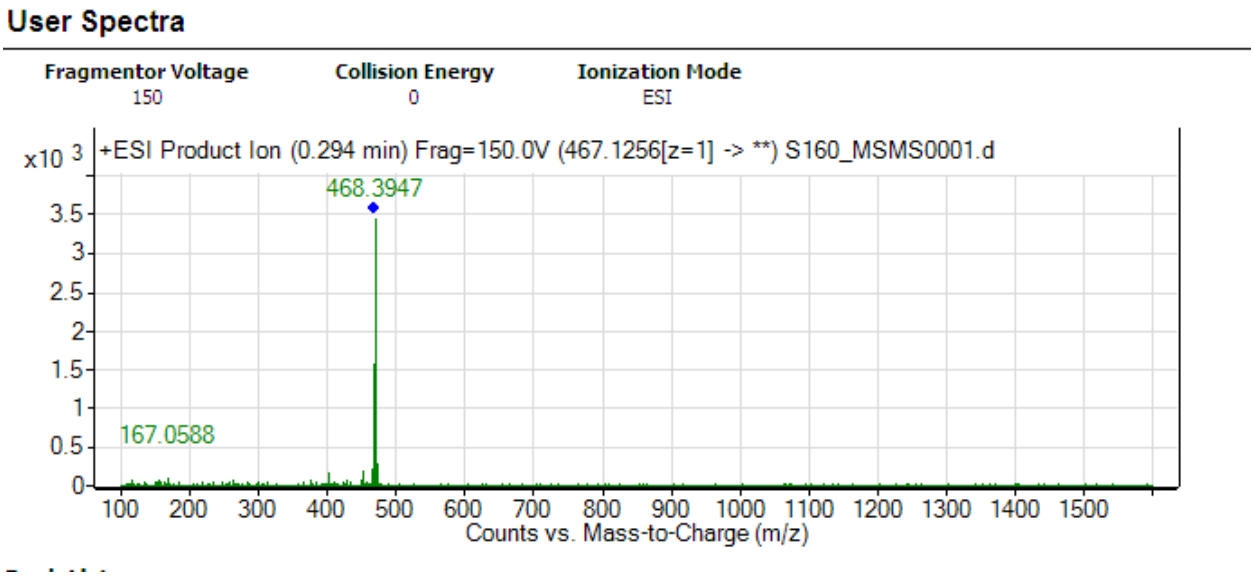


Figure 4.11: LC/MS spectrum of ATQ ProD1.

The figure shows the LC/MS result of ATQ succinate prodrug; molecular formula (positive mode) *m/z* 468 (M+\1), that has a M.W OF 467 g/mol. LC//MS shows that the proposed compound has a M.W of 468g/mol.

An additional confirmation of the identity of the proposed compound is HPLC assessment that was made as a complementary analysis in order to reveal the behavior of the drug and the prodrug at certain chromatographic conditions.

The chromatograms of ATQ and ATQ prodrug are shown in the section below.

4.2 HPLC ANALYSIS

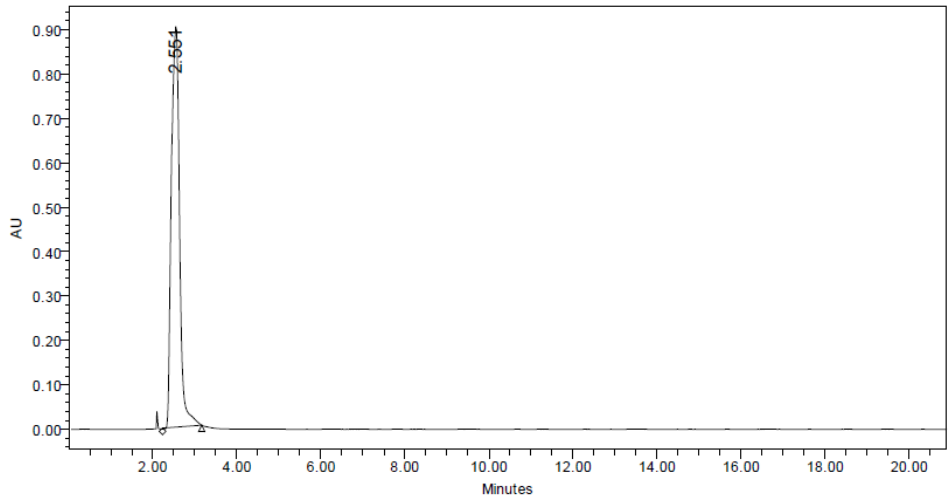


Figure 4.12: ATQ chromatogram.

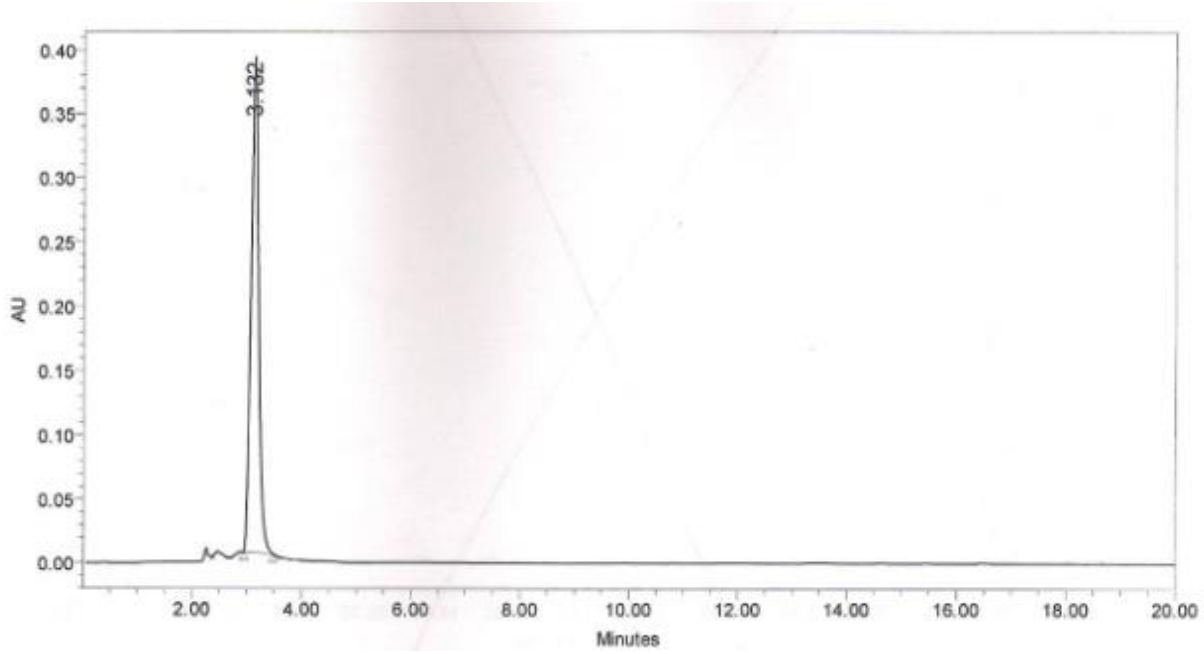


Figure 4.13: ATQ succinate prodrug chromatogram.

The chromatograms shown above are for ATQ and ATQ succinate prodrug, respectively.

The chromatograms ensure the presence of two different pure compounds, as the retention time of ATQ is 2.55 mins while for ATQ prodrug is 3.1 mins.

Altogether, the results that are shown previously indicate that ProD1 is successfully synthesized and purified, and that the recorded data are consistent with the proposed compound.

4.2.1 Kinetic studies on the stability of ATQ ProD1 at different buffer conditions

Conversion time into the parent drug in aqueous media was evaluated .This was carried out by evaluating chemical hydrolysis of the prodrug at pH2.2, pH 5.2 and pH 7.4 solutions using HPLC. These conditions are corresponding to the pH conditions of the stomach (the pH of the stomach in the fasted state ranges from 1.2 to 6.4, median 1.7, and in the fed state it reaches 7, and then decreases gradually within 1.5 hrs) [67], pH of the small intestine and the pH of the blood, respectively. Injections into HPLC apparatus was made after sample preparation at (zero time,1,2 ,3,4,5 ,24 hrs, then after one weak and so on, till 50% prodrug degradation took place.

4.2.1.1 Chemical hydrolysis (release kinetic studies)

It will be clear in the following sections that at constant pH and temperature the reaction displayed strict first order kinetics as the k_{obs} was fairly constant and a straight plot was obtained on plotting log concentration of residual prodrug *vs.* time. The rate constant (k_{obs}) and the corresponding half-lives ($t_{1/2}$) for atovaquone ProD 1 in the different media were calculated from the linear regression equation correlating the log concentration of the residual prodrug *vs.* time, table (4.3).

Table 4.3: A summary of $t_{1/2}$ of ProD 1at the listed pH values.

pH value	T1/2	K_{obs}
pH 2.2	693 hrs (28.8 days)	0.001
pH5.2	53.3 hrs (2.2 days)	0.013
pH7.4	77 hrs (3.2 days)	0.009

Table 4.4: Shows the major chromatographic parameters.

Chromatographic parameters	***pH 2.2***	***pH 5.2***	***pH 7.4***
Rs (resolution)	*0.932*	*0.78*	*1.67*
α (selectivity)	*1.39*	*1.84*	*1.82*
K_{ATQ} (capacity factor for ATQ)	*1.4*	*1.44*	*1.72*
K_{proD1} (capacity factor for ATQ prodrug)	*1.97*	*2.66*	*3.1*

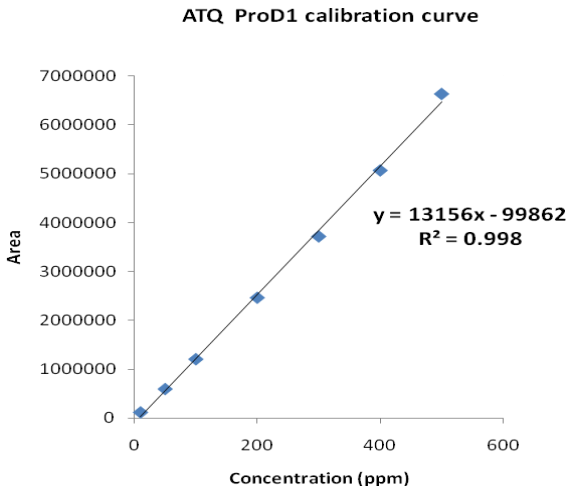


Figure 4.14: Shows the calibration curve of ATQ ProD1.

4.2.1.1.1 ATQ ProD1 hydrolysis at pH 2.2

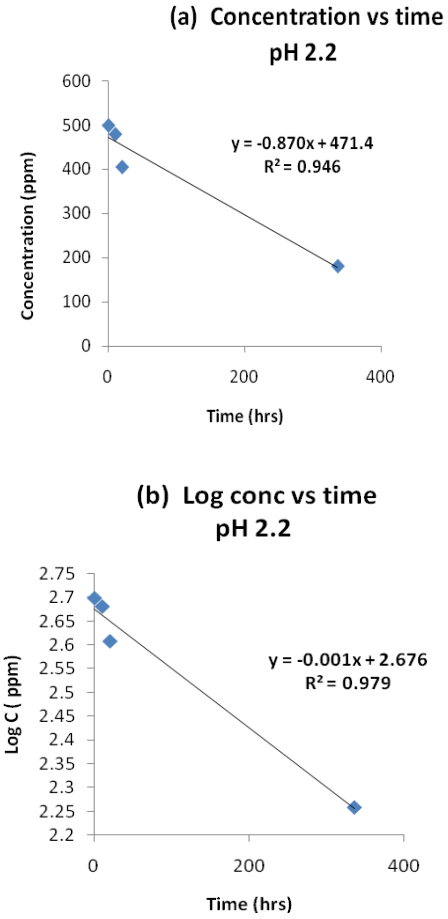
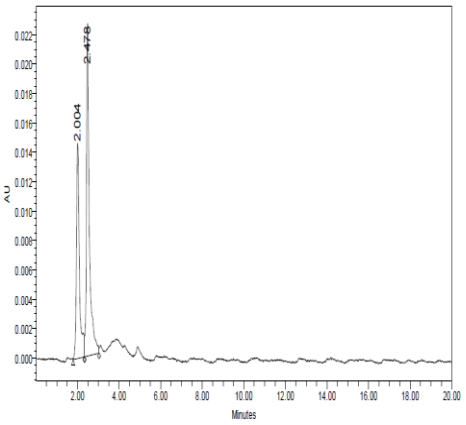
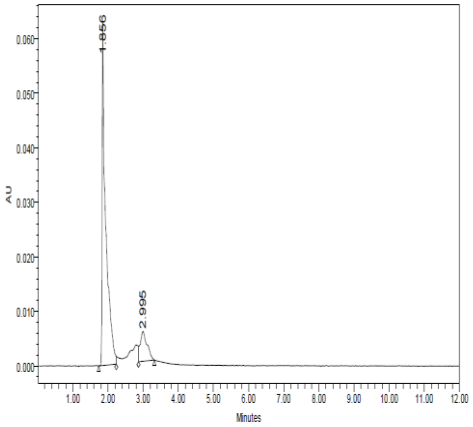


Figure 4.15: first order hydrolysis of ATQ Succinate prodrug at pH 2.2



	RT	Area	% Area	Height
1	2.004	147221	40.28	14640
2	2.478	218272	59.72	22625



	RT	Area	% Area	Height
1	1.851	260464	77.65	18481
2	2.942	74951	22.35	5748

Figure 4.16: ATQ succinate prodrug chromatogram at t=9 hrs and t= 336hrs (14 days), respectively.

4.2.1.1.2 ATQ Succinate prodrug hydrolysis at pH 5.2.

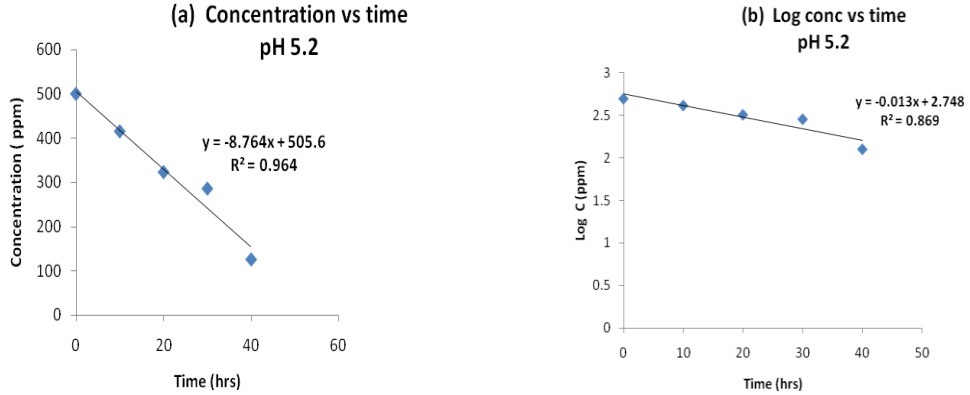
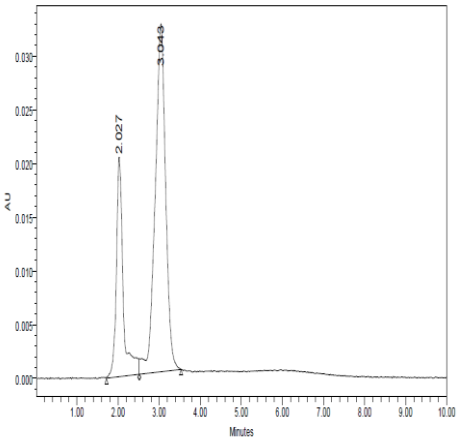
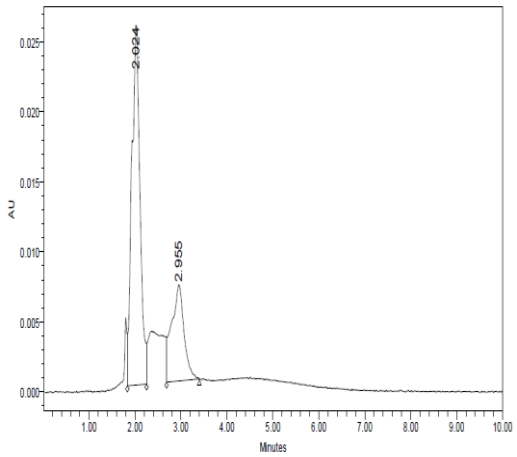


Figure 4.17: shows the behavior of ATQ Succinate at pH 5.2.





	RT	Area	% Area	Height
1	2.027	235568	29.02	20523
2	3.043	576049	70.98	32466

	RT	Area	% Area	Height
1	2.024	316374	72.81	25696
2	2.955	118169	27.19	6532

Figure 4.18: ATQ Succinate prodrug pH5.2, t=3 hrs and t= 40 hrs, respectively

4.2.1.1.3 ATQ Succinate prodrug hydrolysis at pH 7.4.

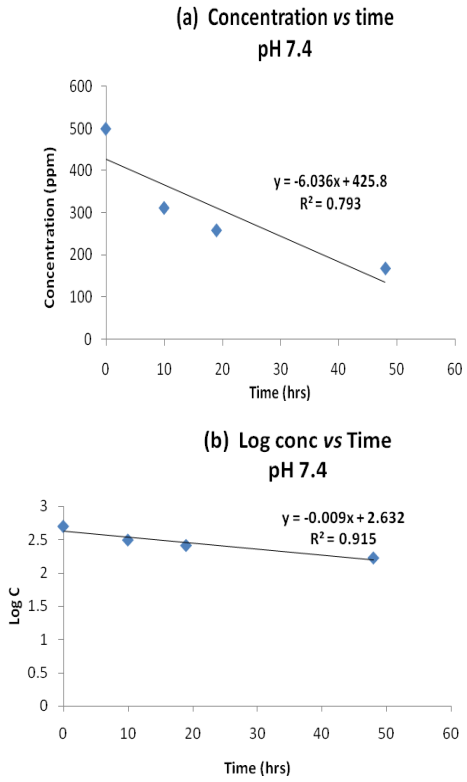
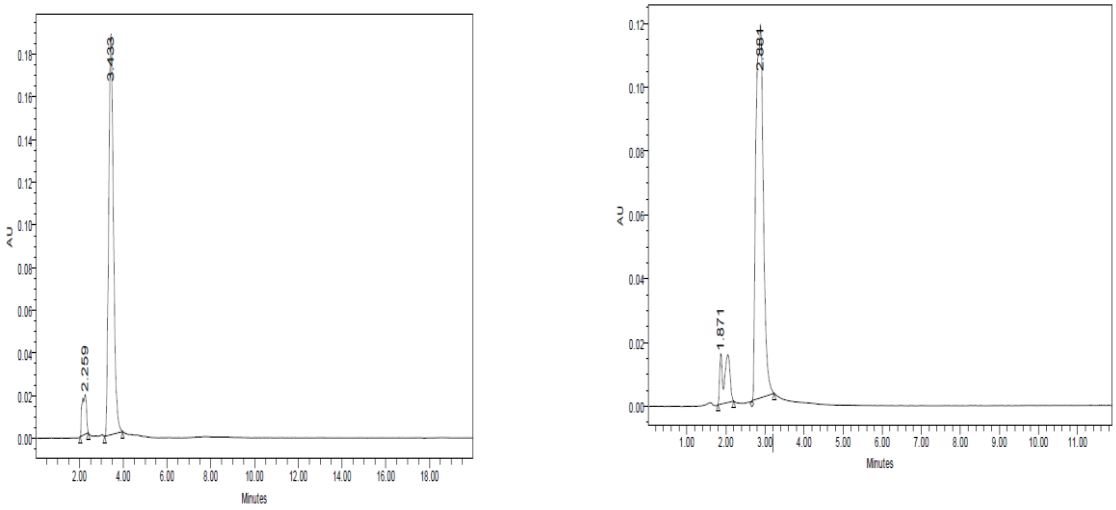


Figure 4.19: first order hydrolysis ATQ Succinate hydloysis at pH 7.4.



	RT	Area	% Area	Height
1	2.259	258865	8.33	18462
2	3.433	2847832	91.67	187458

	RT	Area	% Area	Height
1	1.871	203597	11.64	15915
2	2.881	1545007	88.36	116647

Figure 4.20: ATQ Succinate prodrug at 7.4 after 3 hrs and t=8 days, respectively.

According to Bruice proposed mechanism of dicarboxylic acid semi ester hydrolysis, and based on the results listed in table (4.3), it can be concluded that ATQ prodrug hydrolysis rate is increased as the pH of the medium increased (comparing the results at pH 2.2 *vs.* the results at pH 5.5 and 7.4). This finding can be explained based on Bruice mechanism of intermolecular cyclyzation, which stays that as the pH of the medium is increased the ring closing reaction rate will increase.

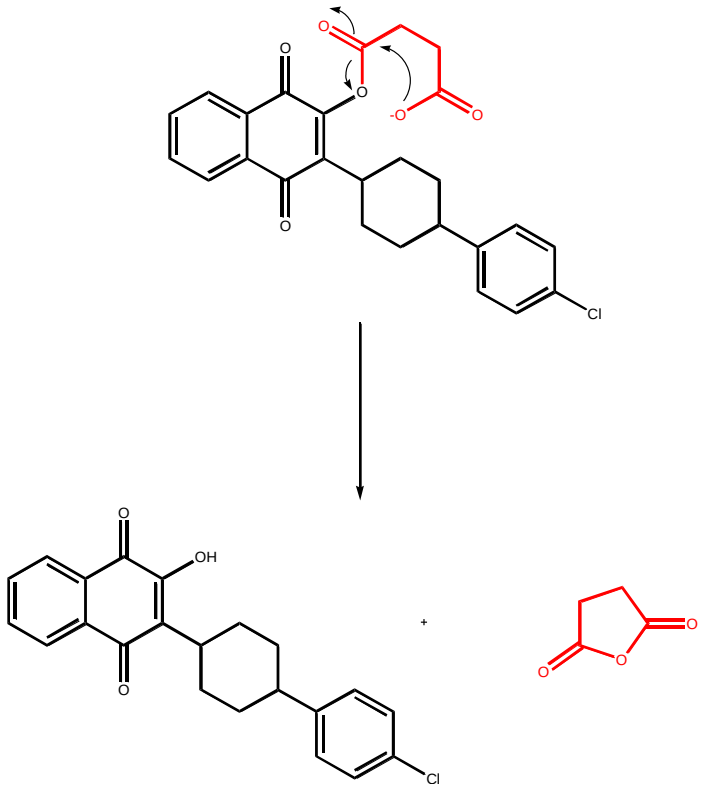
This is because at basic pH the OH group of the succinate part becomes deprotonated, consequently the nuceophilicity of this group will be enhanced, and this facilitates the nucleophilic attack of the OH group on the electrophile center, thus the ring closing reaction will be completed and the parent drug will be elaborated, scheme (4.1).

To translate these findings into practice, an intravenous and oral administration of ATQ succinate prodrug is possible.

The controlled release in combination with the long t1/2 of ATQ, allows for administering the drug once daily. This contributes in improving patient compliance and hence treatment outcomes. Furthermore, the controlled release of the parent constitutes a major advantage for highly lipophilic drugs like ATQ, since a rapid cleavage leads to drug precipitation and poor re-dissolution [68], figure (4.23).

It is important to mention that succinic anhydride is particularly considered suitable linker, because it is produced in the body in cribs' cycle, so it is non toxic when released *in vivo* after prodrug hydrolysis, and it becomes ionized at physiological pH, so it contributes to the increased solubility of the prodrug.

Altogether, the idea of simple administration of effective drugs that treat endemic diseases like malaria becomes feasible and applicable.



Readily permeable once cleaved.

Scheme 4.3: Shows the hydrolysis of ATQ succinate in the body.

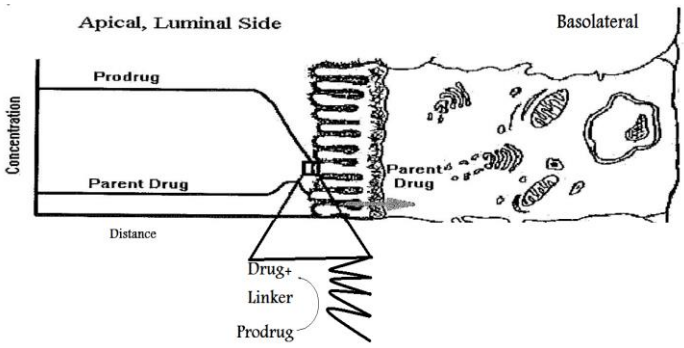


Figure 4.21: Shows the hydrolysis and absorption of prodrugs in the GIT.

Conclusions and Recommendations

Chapter 5

Conclusions and recommendations

5.1. Conclusions

Malaria is an endemic global disease. Malaria control programs need efficacious drugs that can be used with ease by the populations of endemic countries.
Several medicines are used to treat malaria; however, the limited efficacy, severe side effects and the appearance of parasitic resistance limit their use.

Several lines of evidence indicate that the emergence of drug resistant parasites stimulate research groups to explore different approaches in an attempt to improve the characteristics of existing medications.

ATQ, a naphthoquinone and ubiquinone analogue seems to be promising in the treatment and prevention of malaria. It is an effective drug, it inhibits electron transport in the parasite leading to its death, it has an excellent safety profile, and there is no registration of side effects that obligate stopping of therapy, it has long half life and it can be taken orally. Nevertheless, it has bad bioavailability and it is expensive.
ATQ formulation improvements is done and showed better bioavailability than conventional tablet dosage form.
Prodrugs, an alternative approach lead to improved efficacy, pharmacokinetics profile and reduced toxicity. Additionally, prodrug approach saves money and time which is an important issue in drug development. ATQ prodrug 17C91 show a 3 fold increase in bioavailability over the improved ATQ formulation (micronized suspension) and conventional tablets, meaning that ATQ prodrugs are successful.
Our research is complementary to prodrug approach, this research come out with a new entity that possesses superior properties over ATQ and existing ATQ prodrugs, in terms of efficacy, safety, physicochemical properties and drug releasing rate. Consequently, ATQ succiate prodrug is the desired compound that fulfills the requirements of malaria treatment with a standard medication like ATQ. Such effort may has implication on other studies aim to develop prodrugs for other antimalarial classes.

5.2. Recommendations and future directions

To overcome or decrease drug resistance, we recommend using the right effective medication for the people who need it. Our future directions is to evaluate if our produced prodrug has any antimalarial activity, in addition to the performance of *in vivo* studies of ATQ succinate prodrug in both animals and humans, this is most likely will be done in Finland.

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تطوير دواء اولي مبتكر للاتوفاكوون; الدواء المضاد للملاريا: تصنيع كيميائي, تشخيص و تقييم القوى المحركه المخبريه.

إعداد الطالبة: بيسان وضاح امين الفتاش.

المشرف الرئيسي: بروفيسور رفيق قرمان.

الملخص:

يعتبر مرض الملاريا من الامراض الصحيه العالميه, والتي تتسبب بمليون حالة وفاه سنويا, يشكل الاطفال تحت عمر ال 5 سنوات نسبه كبيره منها. وتعزى معظم حالات الوفاه الى اكثر اشكال المرض حده والتي تسببها بلازموديوم فالسيبارم احد الطفيليات المسببه للملاريا.

يتوفر عدد من العلاجات الفعاله لهذا المرض الطفيلي, الا ان الطفيل المسبب للمرض يطور مقاومه ضد هذه الادويه مما يقلل من فعاليتها ضده, مما شكل خطوره في السيطره على هذا المرض الوبائي. ونيجة لذلك تم تكثيف الجهود نحو تطوير ادويه ذات تاثير فعال ضد مرض الملاريا, ومن ضمن هذه العلاجات دواء الاتوفاكوون , والذي يعتبر من الادويه الحديثه نسبيا و التي ساهمت في تقدم علاج الملاريا.

اتوفاكوون يتسم بصفات ايجابيه عديده, الا ان بعض المساوىء المتعلقه به حدت من فعاليته. ومن ابرز هذه السلبيات ذائبية الاتوفاكوون المحدوده في سوائل الجسم وبالاخص عصارة الجهاز الهضمي , والتي تشكل متطلب رئيسي حتى يتم امتصاص الدواء الى الدوره الدمويه يتراكيز كافيه لاظهار فعالية الدواء.

ان النسبه بين كمية الاتوفاكوون التي يتم امتصاصها الى الدم و الجرعه التي اعطيت للمريض تشكل فقط 10% في حالة الصيام. وهذه نسبه ضئيله بحاجه الى التحسين حتى تتم الاستفاده من الجرعه المعطاه بافضل صوره ممكنه.

ومن هنا, تم تحديد هدف عملنا البحثي , وهو العمل على تحسين المبنى الكيميائي للدواء بحيث تتم زيادة قطبية المركب وبالتالي زياة ذائبيته في الوسط المائي. تمت عملية التحسين هذه عن طريق ربط الاتوفاكوون ب سكسينك انهيدرايد, والذي يشكل الجزء القطبي, عن طريق تفاعل كيميائي, ليتم انتاج دواء اولي.

عمليات التنقيه للمركب الناتج تشمل, استخلاص للمركب الناتج من باقي نواتج التفاعل, واعادة بلورته, واستخدام تقنية Column chromatography ال للحصول على مركب نقي.

تم التاكد من نقاء المركب باستخدام تقنية ال HNMR, IR, HPLC.

تم تقييم العمر النصفي للمركب في المحاليل التاليه, ذات درجات الحموضه المختلفه:

pH 2.2, 5.5, 7.4,

ووجد ان العمر النصفي في هذه المحاليل هي كالتالي:

28,8ايام ,2.2 ايام ,3.2 ايام, على التوالي.

حيث انه يتم اطلاق الاتوفاكوون من الدواء الاولي بشكل يعتمد على درجه حموضة المحلول, تحت درجة حراره ثابته. كما ان الرسم الباني لهذه النتائج قد اعطى خط مستقيم, مما يدل على وجود قوى محركه من الرتبه الاولى.

وبهذا, تم الاستنتاج ان التعديل على دواء الاتوفاكوون قد ساعد في زيادة ذائبيته في الوسط المائي بالتالي تحسين امتصاصه من

الجهاز الهضمي, ونتيجة لذلك تحسن في فعليته في علاج مرض الملاريا الوبائي, اضافة الى المساعده في تشكيل الدواء باشكال دوائيه مختلفه.